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IpaD is localized at the tip of the *Shigella flexneri* type III secretion apparatus

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Abstract

Type III secretion (T3S) systems are used by numerous Gram-negative pathogenic bacteria to inject virulence proteins into animal and plant host cells. The core of the T3S apparatus, known as the needle complex, is composed of a basal body transversing both bacterial membranes and a needle protruding above the bacterial surface. In *Shigella flexneri*, IpaD is required to inhibit the activity of the T3S apparatus prior to contact of bacteria with host and has been proposed to assist translocation of bacterial proteins into host cells. We investigated the localization of IpaD by electron microscopy analysis of cross-linked bacteria and mildly purified needle complexes. This analysis revealed the presence of a distinct density at the needle tip. A combination of single particle analysis, immuno-labeling and biochemical analysis, demonstrated that IpaD forms part of the structure at the needle tip. Anti-IpaD antibodies were shown to block entry of bacteria into epithelial cells.

Keywords: IpaD; Needle complex; Type III secretion system; *Shigella flexneri*; Electron microscopy

1. Introduction

Many Gram-negative pathogenic bacteria use a type three secretion (T3S) system to interact with cells of their host. Each T3S system consists of a secretion apparatus (T3SA) that spans the bacterial envelope and extends on the bacterial surface, translocators that transit through the T3SA and insert into the membrane of the host cell where they form a pore, effectors that transit through the T3SA and the translocator pore to reach the cell cytoplasm, specific chaperones that associate with translocators and effectors in the bacterial cytoplasm and transcriptional regulators. Approximately 15 proteins are required for assembly of the T3SA.

Bacteria belonging to the *Shigella* genus are the causative agents of bacillary dysentery in humans [1]. Genes required for entry of bacteria into epithelial cells and inducing apoptosis in macrophages are clustered in a 30-kb region, designated the entry region, of a 220-kb virulence plasmid. The entry region contains 20 *mxi* and *spa* genes encoding components of the T3SA, the *ipaA*, *B*, *C* and *D*, *ipgB1*, *ipgD* and *icsB* genes encoding proteins that transit through the T3SA, the *ipgA*, *ipgC*, *ipgE* and *spa15* genes encoding chaperones, and the *virB* and *mxiE* genes encoding transcriptional regulators [2].

The T3SA, which is weakly active in bacteria growing in broth, is activated upon contact of bacteria with epithelial cells [3]. Inactivation of *ipaB*, *ipaC* or *ipaD*, as well as most *mxi* and *spa* genes, abolishes the ability of bacteria to enter epithelial cells, induce apoptosis in macrophages and express contact hemolytic activity. IpaB and IpaC contain hydrophobic segments and remained associated with the membrane of lysed erythrocytes, suggesting that these two proteins are components of the *S. flexneri* translocator. In addition, effector functions have been proposed for IpaB and IpaC [4–8]. Inactivation of *ipaB* and *ipaD*, but not *ipaC*, leads to a deregulated, i.e. constitutively active, T3SA, suggesting that IpaB and IpaD play a role in maintaining the T3SA inactive in

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the absence of inducers [9,10]. A small proportion of IpaD is associated with the bacterial envelope [9,11]. Picking and collaborators [12] reported that the role of IpaD in the control of the T3SA activity can be separated from its role in entry of bacteria into epithelial cells.

To get further insights on the structure of the needle complex, we performed an immuno-electron microscopic analysis on bacteria treated with the cross-linking agent BS^3, both on entire bacteria and on the mildly purified needle complex (NC). We present evidence that IpaD is a component of the NC localized at the tip of the needle and that antibodies raised against IpaD have an inhibitory effect on entry of *S. flexneri* into epithelial cells.

2. Materials and methods

2.1. Bacterial strains and growth media

Strains used in this study are the wild-type *S. flexneri* 5 strain M90T-Sm [13], its *ipaD* derivative SF622 [14]. Bacteria were grown in tryptic casein soy broth (TSB) (Sigma) at 37 °C.

2.2. Purification of NC

NCs were purified as described [15]. Bacteria in the exponential phase of growth in 1 l of TSB at 37 °C were collected by centrifugation, resuspended in 25 ml of phosphate-buffered saline and incubated in the presence of 1 mM Bis (Sulfosuccinimidyl)suberate (BS^3) for 30 min at 37 °C. The mixture was supplemented with 100 mM Tris–HCl and incubated for 15 min at 37 °C. BS^3-treated cultures were harvested and resuspended in an ice-cold lysis buffer (0.5 M sucrose, 20 mM Tris–HCl [pH 7.5], 2 mM EDTA, 0.5 mg/ml lysozyme) supplemented with 1 mM phenylmethylsulfonyl fluoride and incubated for 45 min at 4 °C and for 15 min at 37 °C. Resulting spheroplasts were incubated with 0.01% Triton X-100 for 30 min and treated with 4 mM $MgCl_2$ and 80 μg/ml DNAse (Sigma) for 20 min at 30 °C. Debris were removed by centrifugation (20,000 × *g* for 20 min at 4 °C) and the membrane fraction was pelleted by centrifugation (110,000 × *g* for 30 min at 4 °C) and resuspended in TET buffer (20 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.01% Triton X-100). Immunoblotting analysis was performed with antibodies raised against MxiJ, MxiN and IpaD as described [16].

2.3. Electron microscopy and image analysis

Whole cells and samples of purified NCs were negatively stained with 2% uranyl acetate on glow discharged carbon-coated copper grids. Electron microscopy was performed on a Philips CM120FEG equipped with a field emission gun operated at 120 kV. Images were recorded with a 4000 SP 4 K slow-scan CCD camera at 80,000× magnification at a pixel size (after binning the images) of 3.75 Å at the specimen level, with "GRACE" software for semi-automated specimen selection and data acquisition [17]. Single particle analysis including multi-reference and non-reference procedures, multivariate statistical analysis and classification was performed as described [15]. For immuno-labeling, purified NCs were incubated with affinity purified IpaD polyclonal antibodies (pAbs) at a final concentration of 0.132 ng/μl) for 1 h at 20 °C. Samples were stained with 2% uranyl acetate and observed as above.

2.4. Invasion assay

Two ml of cultures of wild-type or *mxiD* strains in the exponential phase of growth ($OD_{600\ nm}$ of 0.4) were washed twice with 2 ml EBSS before incubation. They were incubated in the presence of serum from rabbits immunized against IpaD (anti-IpaD dilution 1/2000:0.132 ng/μl to 1/50: 15.6 ng/μl) or anti-IpaB (1/50) (same concentration) antibodies for 1 h at 37 °C and bacteria were centrifuged on plates containing 2×10^5 HeLa cells for 10 min at 2000 × *g*. After 1 h incubation at 37 °C, cells were washed three times with 2 ml EBSS and incubated during 1 h with 2 ml MEM milieu containing 50 μg/ml gentamycin. After three washes with 2 ml EBSS, plates were incubated with a solution of deoxycholate 0.5% for 15 min at 20 °C and cell lysates were diluted and plated on agar plates for colony counting.

3. Results

3.1. A distinctive structure at the tip of the T3SA needle

Protein purification procedures tend to select for most stable complexes that might not contain weakly associated subunits. We recently showed that a Triton-X100 detergent concentration as low as 0.01% was sufficient to induce the release of NCs from the membrane [15]. To detect potentially labile subunits attached to the needle, we performed a cross-linking step with BS^3 on bacteria prior to any purification. Electron microscopy analysis indicated that, following BS^3 treatment, most bacteria exhibited needle appendages with an additional density at the extremity of the tip (Fig. 1).

NCs were purified from BS^3-treated bacteria after detergent solubilization of membranes as described [18]. Preparations contained a sufficient number of NCs with the additional densities at the needle tip to perform a structural analysis. To

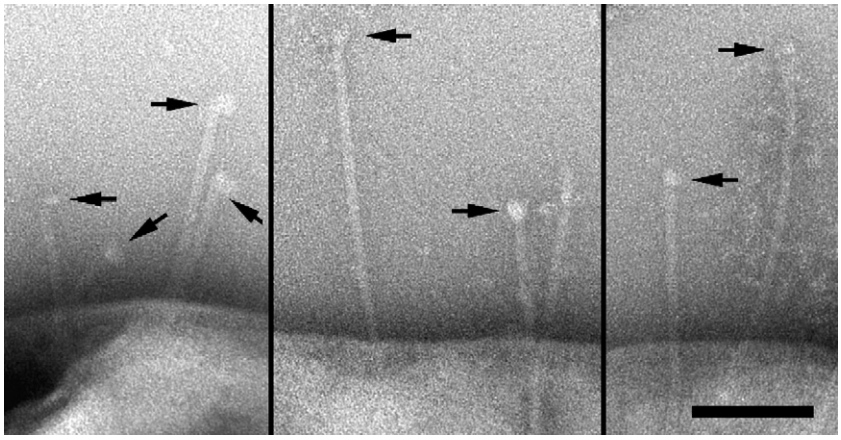

Fig. 1. Electron micrographs of negatively stained bacteria treated with BS^3. Arrows indicate distinct densities at the tip of needles protruding from the bacterial surface. The bar represents 100 nm.

calculate two-dimensional projection maps of isolated NCs, electron microscopy images were analyzed by single particle analysis. We selected several hundred images of NCs with a relatively straight and short needle appendage and a length close to 45 nm. The averaged NCs clearly showed the presence of a density around the needle tip, as well as the upper part of the basal body (Fig. 2A and B; see also Fig. 4E for a total view of a NC). However, NCs appeared a bit blurred after averaging as a result of variations in the needle length. Sharper features at the tip of the needle portion were obtained when projections were aligned and classified after masking the basal part (Fig. 2C). Striking features of the average map of cross-linked particles are the presence of densities at either side of the needle tip. In contrast, average maps of particles prepared from the wild-type strain without cross-linking showed needles lacking most of these densities (Fig. 2D–F). Faint densities are visible in these samples at the same position where the strong densities were present in cross-linked preparations (arrows, Fig. 2E and F). These results suggest that, in the absence of cross-linking, most purified NCs lost the additional molecule(s) forming the density observed after cross-linking. To identify molecule(s) forming the density at the tip of the T3SA, we performed similar experiments with an *ipaD* mutant lacking the IpaD expression.

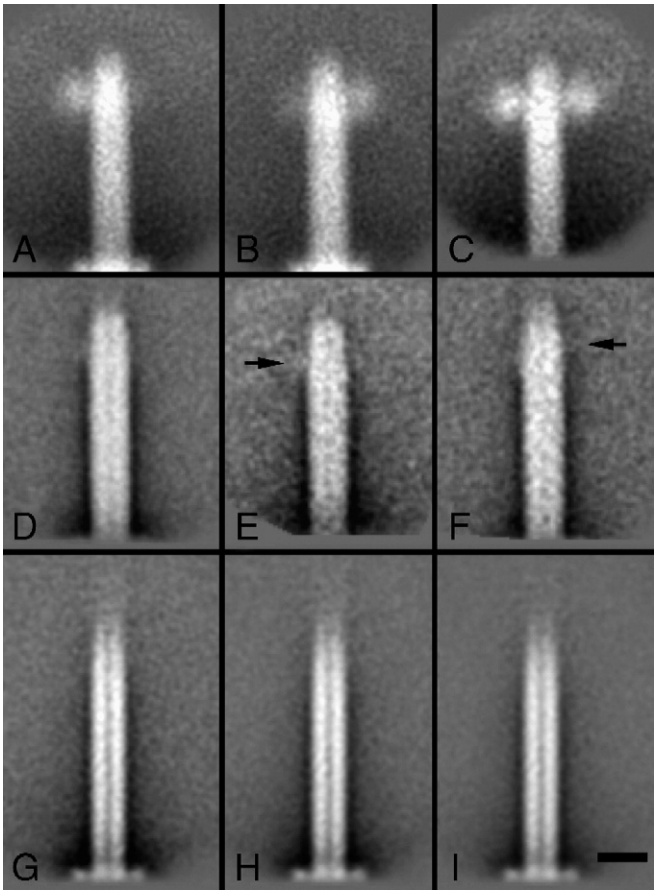

Fig. 2. Projection maps (class-sums) of the needle part of NCs obtained by single particle analysis. (A–B) maps of NCs isolated from wild-type, treated with BS^3 (A–C); from wild-type without BS^3 treatment (D–F) and from an *ipaD* mutant strain with BS^3 treatment (G–I). The arrows point to remnant densities attached to needle parts prepared without BS^3-treatment. The bar represents 10 nm.

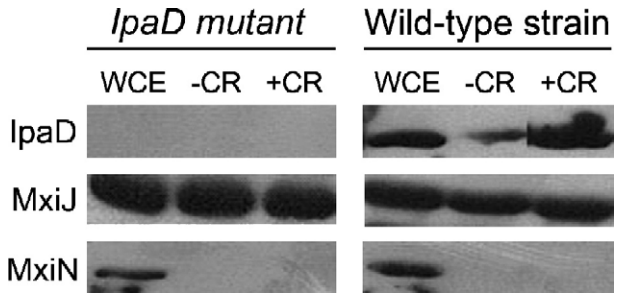

Fig. 3. (A) Immunobloting analysis of purified NCs. Whole cell extracts (WCE) and cross linked (+CR) and non-cross linked (−CR) NCs purified from wild-type and IpaD deficient strains were analyzed by SDS-PAGE and immunoblotting using antibodies specific to MxiJ, MxiN, and IpaD. IpaD was only enriched to WCE level in NCs prepared from cross-linked wild-type bacteria. MxiJ is a positive control to demonstrate intact T3SA. MxiN, a cytoplasmic component of T3S, is a positive control to demonstrate contamination by non-bound cytoplasmic proteins.

IpaD is required, together with IpaB, to maintain the T3SA inactive in the absence of inducers and a small proportion of IpaD is membrane associated [9]. NCs purified from the BS^3-treated *ipaD* mutant did not exhibit densities at either side of the needle tip (Fig. 2G–I), suggesting that IpaD is part of or required for assembly of this structural element.

3.2. IpaD is present at the tip of the T3SA needle

To test whether IpaD constitutes the observed density, NCs purified from BS^3-treated wild-type bacteria were analyzed by SDS-PAGE and immunoblotting (Fig. 3). IpaD was enriched in

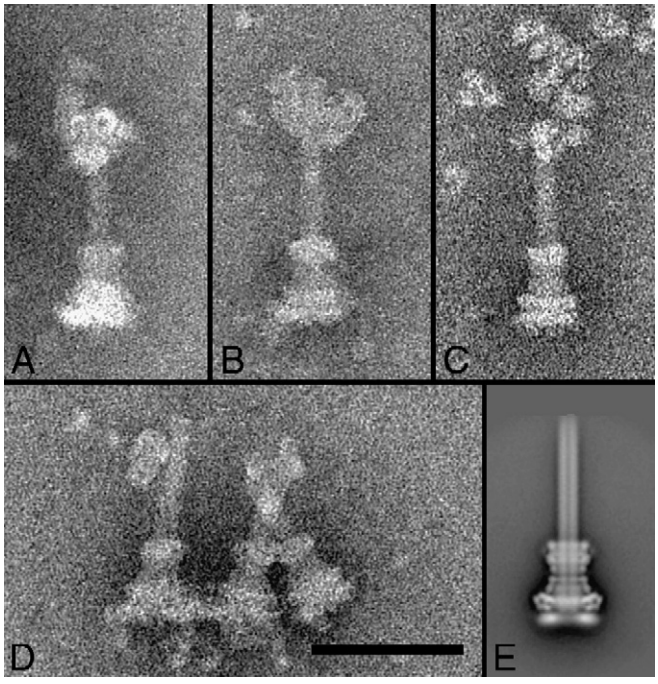

Fig. 4. IpaD localization by immuno-electron microscopy. NCs purified from BS^3-treated wild-type bacteria were incubated with anti-IpaD antibodies and negatively stained (A–D). The average image of 250 NCs purified from bacteria not treated with BS^3 is shown in panel E for comparison. The bar represents 10 nm.

NCs prepared from cross-linked wild-type bacteria, as compared to NCs prepared from non-cross-linked bacteria (Fig. 3, right panel, right lane), though small amounts also co-purify in non-cross linked preparation and thus corroborate the faint densities at the needle tip for averages of non-cross linked NCs. MxiJ that is a major NC component is present in similar amounts in all preparations (Fig. 3). Control experiments using antibodies recognizing cytoplasmic components of the T3S system, such as MxiN, did not reveal any contamination of NCs by intracellular components (Fig. 3), indicating that the presence of IpaD in the preparations was not due to a contamination by cytoplasmic proteins.

To confirm that densities detected at the tip of NCs contained IpaD, we performed immuno-staining using an anti-IpaD serum. Antibodies specifically bound to the tip of the needle in NCs prepared from BS^3 treated wild-type bacteria (Fig. 4A–D). Some needles were also observed to be associated by their tip, presumably as a result of the interaction to bivalent antibodies with two needles (lower left frame, Fig. 4D). In control experiments, no antibodies were found to bind the needle of NCs isolated from the *ipaD* mutant (data not shown).

3.3. Anti-IpaD antibodies blocks entry of bacteria into epithelial cells

Since IpaD is required for entry of bacteria into epithelial cells [14] and since, as shown here, it is localized at the tip of the T3SA, we investigated whether anti-IpaD antibodies might interfere with entry of bacteria into HeLa cells. Bacteria incubated with different concentrations of the anti-IpaD serum, or an anti-IpaB serum as a control, were used to infect HeLa cells. Exposure of bacteria to the anti-IpaD serum, but not to the anti-IpaB serum, inhibited bacterial entry in a dose-dependent manner (Fig. 5). Treatment with the anti-IpaD antibodies also inhibited entry of a *S. flexneri 2a* strain (data not shown).

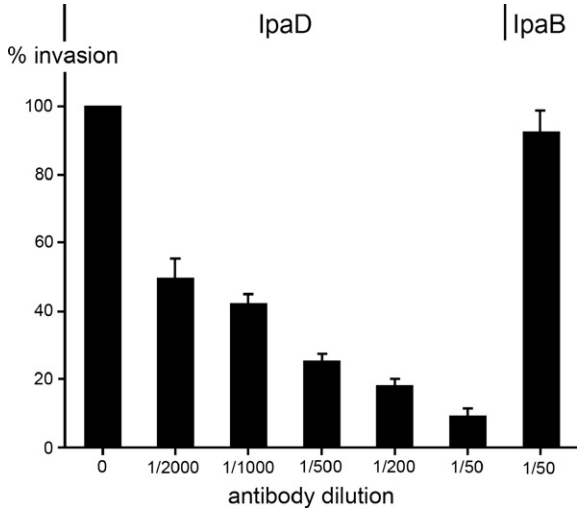

Fig. 5. Invasion assay of epithelial cells by wild-type *S. flexneri*. Bacteria were incubated with serial dilutions of the anti-IpaD or anti-IpaB polyclonal antibodies and intracellular, gentamycin-resistant bacteria were counted by plating cell lysates. The efficiency of entry in each condition is expressed with respect to that of the wild-type strain treated with PBS. The values are the means of at least three independent experiments, and the error bars indicate standard deviations.

4. Discussion

The *S. flexneri* T3SA is activated upon contact of bacteria with epithelial cells and is deregulated by inactivation of *ipaB* or *ipaD*. It was proposed that these proteins are required to form a complex plugging the T3SA. Here, we presented evidence that IpaD is present at the tip of the needle. Transmission electron micrograph of surface exposed needles from cross-linked bacteria showed a distinctive structure present at the tip of the needle and immunoblot analysis of mildly purified NCs indicated that IpaD is copurified with the cross-linked NCs.

Calculated averages of NCs isolated from cross-linked wild-type bacteria showed distinct densities at either sides of the needle tip. This feature was not observed in NCs isolated from both the wild-type strain that had not been treated with the cross-linker and from the *ipaD* mutant treated with the cross-linker. Results of immunoelectron microscopy indicate that the observed density at the tip of the needle contains IpaD molecules. The exact configuration of the additional density, however, cannot be retrieved from 2D projection maps. The two additional masses have dimensions of about 7×7 nm. Since the size of IpaD is 37-kDa, several copies of IpaD are probably present in these structures. Indeed, IpaD has been proposed to form oligomers [19] IpaD presents some functional analogies with LcrV of *Yersinia enterocolitica*, inasmuch as the two proteins have similar sizes and are both required for insertion of the proposed translocators, IpaB and IpaC in *S. flexneri* and YopB and UopD in *Y. enterocolitica*, in the membrane of host cells [9,20,21]. Recent data indicated that LcrV is localized at the tip of the T3SA needle [20]. The structure in *Yersinia* appears to be slightly different to that in *Shigella* with smaller protruding densities at the side and with a different tip.

The identification of a structural element containing IpaD at the tip of the T3SA needle provides further insights on the composition and structure of the *S. flexneri* T3SA. Very recently it was also demonstrated with both biochemical and immunoelectron microscopy methods that IpaD localizes to the T3SA needle tip, where it functions to control the secretion and proper insertion of translocators into host cell membranes [22]. Our single particle analysis, however, directly demonstrates the position of IpaD at the tip of the needle and adds credence to the hypothesis that IpaD acts as a plug to the T3SA prior to contact of bacteria with cells. As proposed for LcrV in *Yersinia*, IpaD might also facilitate insertion of components of the translocators within the cell membrane. The inhibition of entry of bacteria into HeLa cells by treatment with anti-IpaD antibodies indicates that binding of antibodies to IpaD interferes with the function of the protein. LcrV has also been shown to be a protective antigen for plague disease in animal studies [23,24]. Accordingly, IpaD might represent an interesting target for the preparation of vaccines that would be effective against several serotypes of *Shigella*.

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